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Silver enhancement of an immunogold label was used to exhibit the presence of viral antigens in tissue by light microscopy and to identify the intracellular location(s) by electron microscopy. We compared three enhancement procedures: (1) silver lactate reduced by hydroquinone, (2) silver ions in Ilford L4 nuclear research emulsion reduced by metol, and (3) the Janssen IntenSE II enhancement kit. We found the Janssen kit to be the most advantageous for electron microscopy. Keywords:

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LOCALIZATION OF VIRAL ANTIGENS:

IMMUNOGOLD LABELING AND SILVER ENHANCEMENT

OF VIBRATOME TISSUE SECTIONS

M.B. Downs and J.D. White

United States Army Medical Research Institute

for Infectious Diseases, Ft. Detrick, Maryland

In conducting the research described in this report, the
investigators adhered to the "Guide for the Care and Use of
Laboratory Animals", as prepared by the Committee on Care and
Use of Laboratory Animals of the Institute of Laboratory
Animal Resources Commission on Life Sciences-National
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INTRODUCTION

Immunoelectron microscopy (IEM) has become an important
tool for identifying the intracellular sites of specific
proteins. These antigens may be inaccessible to immunologic
markers because they are surrounded by cytosolic proteins and
lipid membranes. Three approaches are commonly used to label
intracellular antigens: (1) post-embedding techniques; or
(2) cryoimmunolabeling, which employ thin sections of
tissue; and (3) pre-embedding techniques, which rely on the
ability of the label to penetrate the tissue.

The post-embedding techniques employ fixation and either
an epoxy resin or a hydrophilic resin such as Lowicryl
K4M[1]. Surfaces exposed during sectioning are stained by
floating tissue-bearing grids on the immunological reagents.
Although post-embedding procedures work well for some
proteins, exposure to fixatives, dehydration chemicals and
the resins can destroy the antigenicity of others. On the
other hand, cryomicrotomy eliminates the need to embed the
tissue; but, to retain the integrity of the tissue during the
immunolabeling procedure, fixation is still necessary[2]. The
main disadvantages of cryotechniques are that cryomicrotomes
are not available in all laboratories and the methodology is
tedious.

In pre-embedding methods, immunological reactions are
carried out between fixation and routine dehydration and

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embedding. These techniques have been used successfully for immunoenzyme techniques, but enzyme reaction products diffuse away from the site of antibody binding and, thus, are of limited usefulness in protein localization[3,4]. In contrast, colloidal gold conjugated to immunoglobulins forms discrete images at the binding site, but does not readily penetrate tissue.

Immunolabeling viral proteins poses special problems. The pattern of viral growth in vitro is distinct from that seen in vivo. Viruses that uniformly infect cultured cells at high titers often grow in only a few cell types in vivo. This makes detection difficult and tissue screening at the light microscopic level necessary. Other factors that may decrease the intensity or amount of labeling to unacceptable levels are the paucity of viral proteins during the early stages of replication and the inactivation of human pathogens for safety reasons.

The ideal immunostain would reach all antigenic sites in the tissue, form discrete images by electron microscopy, and provide enough signal amplification to be visible at low magnifications. This paper describes ultrastructural visualization of labeled intracellular viral antigens in fixed, unembedded, Vibratome-cut, tissue sections with altered permeability, by using small-diameter, immunogold particles bound to specific immunoglobulins. The colloidal gold spheres can be enlarged by silver enhancement for

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screening with the light microscope.

METHODS

FIXATION. Tissue used for silver enhancement of specific immunogold deposits prior to embedding are exposed for extensive times to various, sometimes harsh, reagents. Fixatives must preserve ultrastructural detail and immobilize intracellular proteins without sacrificing antigenicity. Glutaraldehyde fixation gives the best morphological results, but the extensive cross-linkage of proteins often destroys antigenicity. Immunocytochemists originally thought that glutaraldehyde disrupted the tertiary structure necessary for antibody binding[5], but more recent research indicates that aldehyde binding forms a dense meshwork impenetrable to antibodies[3,6]. Satisfactory ultrastructure and antigenicity can be preserved when the minimum concentration of fixative and duration of fixation is determined, empirically, for each antigen-antibody combination. We found that overnight fixation in a mixture of 2 or 4% paraformaldehyde with 0.5% glutaraldehyde buffered in 0.1 M sodium phosphate or sodium cacodylate is suitable for the viral antigens we examined.

VIBRATOME SECTIONING. To facilitate diffusion of the gold-conjugated antibody into the tissue, 40-μm sections are cut by using a Vibratome 1000 (Technical Products International,

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Inc., St. Louis, MO). As the slices are cut, they are transferred, in order, from the Vibratome trough to wells of a tissue culture plate with a sable brush. These tissue slices are stored in a buffer solution of 20 mM Tris-base, 0.9% sodium chloride, 0.1% bovine serum albumin, and 20 mM sodium azide, pH 8.2, until labeling. Tissue slices can be stored at 4°C for an extended time without losing antigenicity; however, ultrastructural morphology will deteriorate because the phospholipids and fats have not yet been stabilized with osmium tetroxide.

SCREENING BY LIGHT MICROSCOPY. Adjacent tissue slices are processed for screening by light microscopy and electron microscopy. After enhancement, an entire 40-μm tissue slice can be viewed by light microscopy to locate foci of silver deposits to use as a guide to locate the same areas in the adjacent section embedded in epoxy resin for EM.

IMMUNOGOLD LABELING. The tissue slices are processed at room temperature in the wells of a porcelain plate placed on a rotator. Agitation of solutions in the wells improves diffusion of the reagents into the tissue. To further enhance the penetration of the reagents into the tissue, all reagents are prepared in Tris buffer (supra vide) to which 0.5% Tween 20 has been added. The standard labeling procedure follows and is diagramed in Figure 1.

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1. Non-specific binding sites are blocked by incubating the tissue for 20 min in a 4% solution of serum from the same species as the secondary antibody (Figure 1a).

2. The normal serum is replaced with primary antibody (Figure 1b). The dilution and time of incubation must be determined empirically for each antibody-virus system.

3. Tissue slices are rinsed three times in Tris-Tween buffer for ten minutes each time to remove unbound antibodies.

4. Tissue slices are incubated for 1 hour in a 1:40 dilution of the secondary antibody conjugated to 5-nm gold (Figure 1c).

5. Slices are rinsed for 10 min in Tris-Tween buffer, followed by three rinses of 3 min each in distilled water to remove all ions prior to silver enhancement.

SILVER ENHANCEMENT. Silver deposits on the colloidal gold when the immunolabeled tissue slices are incubated with a source of silver ions and a physical developer (Figure 1d). Instructions are given for three techniques:

Silver lactate reduced by hydroquinone. This method is modified from Danscher's original technique for light microscopic visualization of metal deposits in tissue[7]. The developer is made by dissolving 0.85 g hydroquinone, 2.55 g citric acid monohydrate, and 2.35 g sodium citrate dihydrate in 85 ml of distilled water. The silver ion supply, which must be protected from light, consists of 0.11

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g silver lactate in 15 ml distilled water. In a photographic darkroom equipped with a Thomas Duplex safelight (Thomas Instrument Co., Charlottesville, VA), the developer and silver donor are mixed and the solution is pipetted into clean wells of a porcelain plate. The tissue sections are transferred to the developing solution with a sable brush. Development requires 3 to 10 min depending on the final grain size desired and is halted by transferring the sections to distilled water.

Ilford L4 reduced by Metol. This technique has been modified from a post-embedding procedure designed for double labeling[9]. The Agfa-Gevaert developer is made by dissolving 75 mg Metol, [4-(methylamino)-phenolsulfate, Fluka Chemical Co., Ronkonkoma, NY], 50 mg sodium sulfite and 20 mg potassium thiocyanate in 10 ml distilled water. The pH is adjusted to 6.3. Silver donor is Ilford L4 nuclear research emulsion (Ilford Limited, Mobberley, Cheshire, England). The Ilford L4 gel is melted for 20 minutes in a 40°C water bath in the dark. After transfer of the tissue to distilled water in clean wells of the porcelain plate, the water is removed, leaving only enough to keep the tissue slices wet. Under a brown safelight (Ilford 902 or Kodak 6B) , 0.3 ml Ilford L4 is mixed with the developer (10 ml) and pipetted over the tissue. After 6-8 min, the tissue slices are removed to distilled water to stop development.

Janssen IntenSE II kit. The IntenSE II kit (Janssen

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Life Sciences Products, Piscataway, NJ) is designed for use at the light microscopic level; however, it works well for pre-embedding IEM. Janssen has formulated the reagents so that development can be done under normal light conditions. The kit consists of two solutions which are mixed just before use. Development, which is best done on a rotator plate, requires 8-10 min for large grains and is stopped by transferring the slices to distilled water.

TISSUE PROCESSING FOR MICROSCOPY

Light microscopy. Tissue slices are post-stained with Mayer's hematoxylin, dehydrated through ethanols and xylene, and mounted with Permount under coverslips on glass microscope slides.

Electron microscopy. Tissue slices are post-fixed with 2% aqueous osmium tetroxide for 30 min, stained en bloc with 0.5% aqueous uranyl acetate for 30 minutes, dehydrated through graded ethanol and propylene oxide and embedded flat in epoxy resin in aluminum weigh boats. Using the adjacent slices processed for light microscopy as a guide, areas of interest are cut from the resin disk with a jeweler's saw. The selected pieces are glued on epoxy blanks and sectioned. The silver label is more easily seen when the tissue is not post-stained.

RESULTS

SILVER LACTATE REDUCED BY HYDROQUINONE. Reduction of silver
lactate by hydroquinone formed grains that were irregular in
shape and size (figure 2A). Development for 7-8 minutes
produced large grains that were seen by light microscopy in
40-μm tissue slices stained with hematoxylin or 1-μm
epoxy-embedded sections stained with toluidine blue, and,
presumably because of the irregular shape, by polarized
light.

ILFORD L4 REDUCED BY METOL. The slow reduction of silver in
the Ilford L4 emulsion by the Agfa-Gevaert developer produced
more spherical grains than the other methods (figure 2B).
Bienz, et al. reported that grains of uniform size are formed
when epoxy sections are incubated for 4-6 min with unmelted
Ilford L4 emulsion and this developer[9]. Enhancement of
immunogold labeled tissue slices for up to 10 min with
unmelted L4 resulted in no staining, while 6-8 minute
development with melted L4 resulted in the formation of
irregularly sized grains. Shorter enhancement times with
melted L4 were not tried. Label developed by this method is
more difficult to see by light microscopy and is not visible
by polarized light.

JANSSEN INTENSE II KIT. Development for 8 min with Janssen

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reagents formed grains which were intermediate in uniformity

between the other methods (Figure 2C). These grains were

easily seen by electron microscopy at low magnification

(Figure 3) and in 40-μm slices prepared for light micro-

scopy. Only areas with very heavy label could be seen

in 1- m epoxy sections. Development for 3 min with the

Janssen reagents resulted in spherical grains which approx-

imately doubled the size of the gold (Figure 4). The

IntenSE II kit has been replaced with the IntenSE M kit for

microscopy and the IntenSE BL for immunoblots. We have not

tested these products for IEM enhancement.

DISCUSSION

Viral antigens are often difficult to detect in tissues

because they are expressed at high titers in only a few

cells. Finding infected cells in tissues labeled with

post-embedding IEM techniques entails electron microscopic

screening large numbers of blocks. In contrast, silver

enhancement of tissue slices labeled with pre-embedding IEM

produced deposits visible with both the light microscope and

the electron microscope. Adjacent tissue slices can be

identically processed through the immunogold labeling step,

then silver-enhanced for light microscopic screening or

antigen localization.

Silver enhancement enlarges the size of the gold label.

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When placed in a solution containing developer and silver ions, metal in tissue catalyzes the reduction of silver ions to metallic silver[7]. Because silver ions are reduced only after being adsorbed on the surface of the catalyst[8], the metallic silver is deposited on the surface of the gold, increasing its size. Thus, small diameter colloidal gold can be used to penetrate the tissue, then is enlarged after intracellular binding.

Selection of the type of enhancement for IEM depends upon the goals of the experiment: relatively large silver grains, visible at low magnifications, are valuable for identifying infected cell types, while smaller grains are better for intracellular protein localization as they do not obscure underlying organelles.

The size and shape of the enhanced gold is dependent on four parameters: time, temperature, silver donor, and developer. The speed of the reaction is directly related to the temperature, so, by controlling time and temperature, the desired grain size can be achieved. However, none of the solutions containing silver ions and developer is stable and the reaction will proceed in the absence of an exogenous catalyst. Therefore, the enhancement must be completed before precipitates form which will deposit on the tissue. A temperature from 21° to 23° is optimal for all enhancement methods and will delay the formation of precipitates until gold-catalyzed grains have formed.

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As the results indicate, the grain shape is a function of the silver donor and developer. Reduction of silver lactate by hydroquinone is the least suitable method for IEM because of the irregularity of the grains. It is, however, the least expensive method and is suitable for large grain development for the identification of infected cells. Reduction of the silver in Ilford L4 emulsion by the Agfa-Gevaert developer forms grains which are spherical, but not uniform in size. The system which Bienz used involved continuous stirring to dissolve the Ilford L4 gel and resulted in grains which decreased in uniformity as the development time increased[9]. The lack of uniformity in size that we found may be due to longer development time, to the lack of agitation of the solution during development, or to the liquid state of the L4. The Janssen IntenSE II solution also forms generally spherical grains, especially during short development times. It is by far the easiest to use because it is not light sensitive.

INTERPRETATION OF RESULTS. As with all immunocytochemical techniques, controls must be used to ensure that silver deposition represents specific antigen-antibody binding. Adjacent slices of infected tissue should be processed by using, as the primary antibody, either (1) an antibody directed against the virus of interest or (2) an antibody directed against a non-related virus. In addition, non-infected tissue slices should be processed using the same

primary antibodies to eliminate the possibility of
non-specific adherence of antibodies to normal tissue
components.

The silver reduction process used for enhancement was
originally designed to identify metals in tissue. Because
any metallic elements will catalyze the reduction of silver,
a tissue slice with no immunolabel should be enhanced to
expose any possible endogenous catalysts. Although tissue
reactivity is rare, this control procedure is especially
important in tissues which can be histologically stained with
silver.

Pre-embedding IEM of permeablized tissue introduces
other possible sources of error which can result in
false-positive or false-negative conclusions. Any pre-embed-
ding technique entails long incubations in the immunological
reagents. Even though the tissue is fixed with crosslinking
agents, some antigen may diffuse during the immunolabeling
process, so ultrastructural antigen distribution is not
necessarily the same as in vivo. Permeabilization has not
been proven to affect all cells uniformly and intact cells
may harbor antigen that is inaccessible to the label.

Finally, because many viral infections are cytolytic,
staining often occurs in areas of necrosis. It is common for
antibodies to non-specifically adhere to necrotic areas. If
labeling is detected in such areas, tissue with a comparable
lesion of different etiology should be stained.

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When the possible sources of error are kept in mind and results are compared with data derived from other techniques, silver enhanced preembedding IEM will offer new insights into the relationships between viruses and cells.

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Figure 1.   Schematic of the silver enhanced immunogold procedure.   a.   Non-specific binding sites are blocked by antibodies from normal goat serum.   b.   Anti-viral mouse monoclonal antibodiese bind to viral antigens.   c.   Goat anti-mouse IgG conjugated to 5-nm gold binds to mouse monoclonal antibody.   d.   The reduction of silver is catalyzed by the gold and it is deposited on the surface of the 5-nm gold particles.
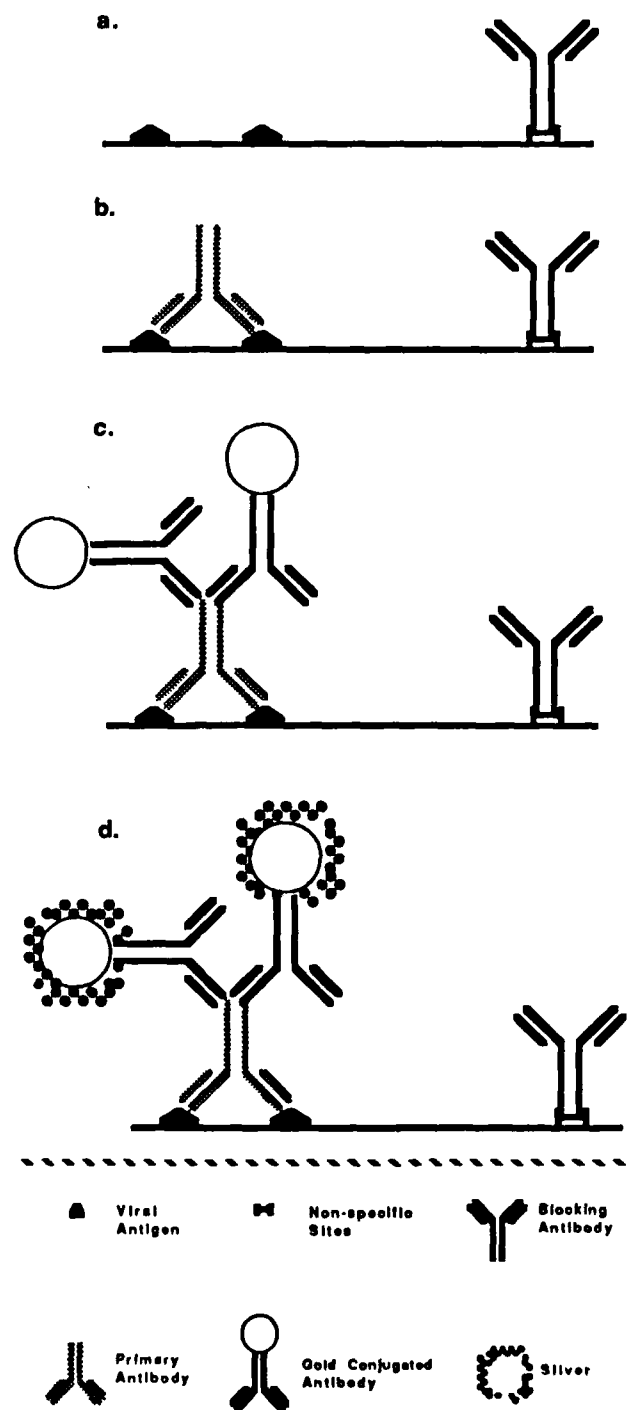
a.

b.

c.

d.

Viral Antigen

Non-specific Sites

Blocking Antibody

Primary Antibody

Gold Conjugated Antibody

Silver

Figure 1

Figure 2. Guinea pig spleen infected with Junin virus and labeled with mouse monoclonal antibody directed against either Junin virus nucleocapsid (a and c) or glycoprotein (b). After incubation with a gold-conjugated secondary antibody, sections were enhanced for 8 min with (a) silver lactate and hydroquinone, (b) Ilford L4 and Agfa-Gevaert developer and (c) Janssen IntenSE II.

Figure 3. Silver-enhanced immunogold label of Junin virus in infected Guinea pig spleen can be seen easily at low magnification, allowing identification of infected cell types.
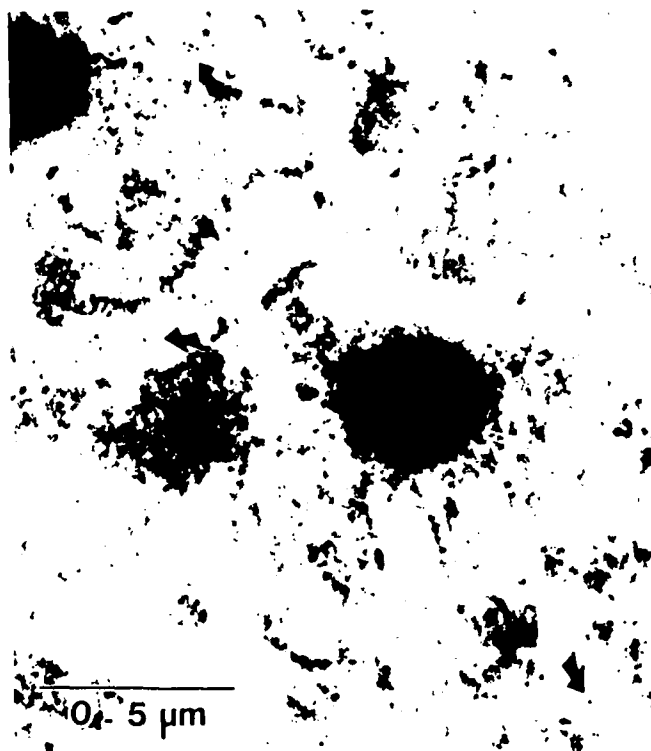
Figure 4. Inclusion bodies in neuron from a mouse infected
with Japanese encephalitis virus. After silver enhancement
of the immunogold by a 3-min incubation with the Janssen
IntenSE II reagents, silver deposits approximately doubled
the diameter of the colloidal gold (arrows).